around Lake Victoria. These doubts could be dispelled by the examination of intermediate populations and the establishment of ranges for each sibling.

It is a reasonable conclusion, therefore, that these four siblings, Sebwe *A*, Sebwe *B*, Nile and Kagera, represent four distinct but closely related species. The sibling identified here as Nile may be *S. damnosum* since the type locality is the 'Jinja fly belt' at the mouth of the Nile. It also raises the possibility that some of Enderlein's names for species in this group may be valid[11,12].

Complete chromosome maps for these four species will be published at a later date. It is also hoped that for this future report it will be possible to examine materials of the *S. damnosum* complex from other parts of Africa.

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PLANARIANS AND MEMORY

By Prof. ALLAN L. JACOBSON, CLIFFORD FRIED and SHELDON D. HOROWITZ

Department of Psychology, University of California, Los Angeles

I. Transfer of Learning by Injection of Ribonucleic Acid

SEVERAL recent investigations of planarians have contributed suggestive evidence regarding the biochemical basis of memory. McConnell[1] and John[2] found that cannibal worms which had ingested classically conditioned worms later performed better than did cannibals which had ingested untrained worms. Corning and John[3] obtained evidence that ribonuclease may 'wipe out' conditioning in regenerating planarians. Two preliminary reports[4] have indicated that injection of ribonucleic acid (RNA) extracted from trained planarians may enhance responding in untrained planarians.

Two related factors have delayed full acceptance of these findings: the controversy concerning the validity of classical conditioning in planarians, and the absence of pseudo-conditioning controls in several of the aforementioned investigations (for discussion of these problems, see ref. 5). The experiment described here was carried out in an attempt to resolve these questions, at least in part, by showing that (*a*) classical conditioning effects can be easily distinguished from pseudo-conditioning effects in planarians, and (*b*) enhanced responding is produced by injection of RNA from classically conditioned but not from pseudo-conditioned or non-conditioned planarians.

675 planarians (*Dugesia dorotocephala*) of about 1 cm length were used, which had been obtained from West Coast Biological Supply Co., Los Angeles. They were maintained and trained in spring water and were not fed during the course of the experiment (the composition of the water, as provided by the laboratory of Sparkletts Drinking Water Corp., was 30 mg/l. calcium, 15 mg/l. sodium, 72 mg/l. chlorine, 12–14 mg/l. bicarbonate, 25 mg/l. silica, and 0·1 mg/l. fluorine). The planarians were handled with a sable brush. The room light was diffused and was on only during the day.

The planarians were trained in a rectangular plastic trough measuring 14·5 cm × 5·5 cm × 5·5 cm and filled with spring water to a depth of 1·5 cm. Aluminium plates which covered the two long walls of the trough served as electrodes. A potential of 10 V peak 60-c/s a.c. was connected to the electrodes by a clock device. Current flow through the volume of water was 2·5 m.amp peak. The conditioned stimulus (CS) was provided by a 75-W bulb in a 25-cm reflector suspended 22 cm above the trough. A piece of paper covered the reflector and diffused the light. The illumination at the trough measured 13 ft.-candles with the CS-light off and 400 ft.-candles with the CS-light on. All stimulus and interval durations were controlled by Hunter clocks.

Three groups of 200 planarians each were used in the first stage of the experiment. One group (non-conditioned, hereafter referred to as group NC) was simply permitted to rest in their home containers. A second group (classically conditioned, or CC) was given training consisting of paired presentations of light and shock. Before these training trials were undertaken, group CC was given fifty 3-sec presentations of light alone in two sets of twenty-five trials separated by a 10-min interval. The interval between light presentations within a set was 20 sec. Then, following another 10-min interval, training was instituted. A training trial consisted of a 3-sec presentation of light, during the last second of which the shock was also applied. The inter-trial interval was 20 sec. On the first day of training, group CC received two sets of twenty-five conditioning trials, with 10-min intervals between adjacent sets, in addition to the fifty light-alone trials. On the next day, group CC received three further sets of training trials.

The third group (pseudo-conditioned, or PC) was trained in the same fashion as was group CC, except that for group PC light and shock were presented in a fixed interspersed sequence, rather than being paired. Whereas group CC received one light-shock pairing every 20 sec, group PC received a stimulus, either light or shock, every 10 sec. The total number of lights and shocks received by group PC in a given trial set was equated to that for group CC, and shock was never given to group PC more than twice in succession. At the beginning of each day's sessions, all worms were adapted to the trough for 10 min before training was commenced.

On each presentation of light, each worm in groups PC and CC was scored according to whether or not it responded during the first 2 sec. A response was defined as a discernible longitudinal contraction of the body, this being the unconditioned response to shock. Other changes in locomotion, including stopping, or lifting, wagging, or turning the head, were not counted as responses. On eight separate occasions, two observers made independent judgments on a set of twenty-five trials. Agreement ranged from 23 out of 25 to 25 out of 25; overall, the two judges agreed on 192 out of 200 trials.

Twenty conditioned and twenty pseudo-conditioned worms (randomly selected) were scored individually for the fifty light trials and 125 subsequent training trials. The performance levels of these animals are presented in Fig. 1. The differentiation between the two groups in their responsiveness to light is apparent and is further indicated by the ranges of scores for the last two training sets: on set 4, the range for CC was 10–17, that for PC 0–5; on set 5, the range for CC was 13–17, that for PC 0–6.

An additional five sets of trials were given to groups CC and PC, three sets on the day preceding extraction (described below) and a final two sets on the day of extraction. During the last trial set, four worms from each of the groups were scored individually. The four CC worms scored 14, 15, 16, 17; the four PC worms scored 1, 2, 3, 3.

After the completion of training, RNA was extracted from each of the three groups of planarians by a phenol-extraction technique (for details of the procedure, see ref. 6). The 200 worms of a given group were pooled together for an extraction. The RNA from each group was dissolved in 700 µl. of isotonic saline. The amount of RNA was determined from the optical density at 260 mµ (ε^p = 7,450 in 0·2 M sodium chloride). One extract was used for the determination. The yield was approximately 3·4 mg/1·0 g of tissue. During the extractions, the experimenters did not know the identity of any group.

Four hours after extraction, the RNA from each of the groups (NC, PC and CC) was injected into twenty-five untrained planarians. Fourteen hours before injection began, each of these 75 recipient worms had been given 100 light-adaptation trials, in two sets of fifty trials separated by a 10-min interval. The inter-stimulus interval within a set was 20 sec. The injections were performed under a binocular dissecting microscope in the following manner: a metal cap was filled with paraffin, and a depression approximately the size of a planarian was cut into the paraffin. The metal cap was placed in a Petri dish and the latter was placed in a container of dry ice. A planarian was placed in the depression. Within 1 min the low temperature resulted in the planarian becoming quiescent. When this was so, 20 µl. of the appropriate solution was injected into the body cavity by way of the pharynx. A 100-µl. No. 710 Hamilton syringe with a 1·3 cm 30-gauge needle was used, and a finely drawn-out capillary tube was glued to the needle. The capillary tube was replaced as necessary during the injections. Like the extractions, the injections were conducted in 'blind' fashion; that is, the research worker carrying out the injections did not know to which group the planarian which he was treating belonged.

Twenty-four hours after the injections had been completed, the 75 injected worms were tested individually. Each worm received twenty-five presentations of light alone, spaced 20 sec apart, and responses occurring during the first 2 sec of the light were recorded. No shock was ever given during testing. Again, all testing was conducted 'blind'.

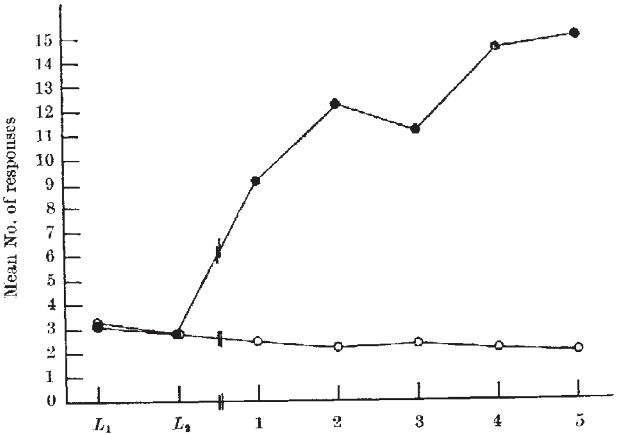

Fig. 1. Mean number of responses per set of 25 trials for groups CC and PC. On the abscissa, L_1 and L_2 are sets in which light alone was presented, and 1–5 represent training sets. ●, CC (N = 20); ○, PC (N = 20)

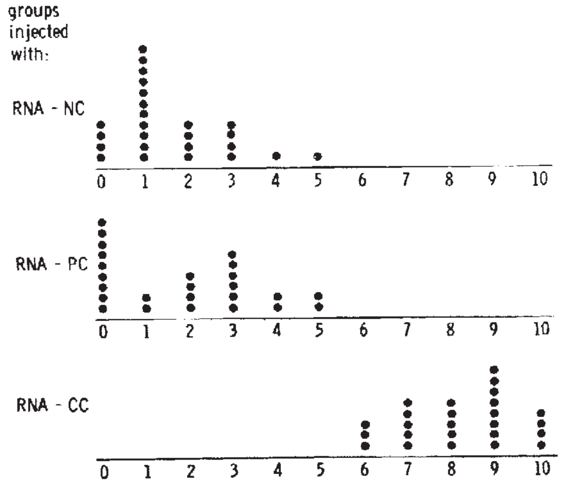

Fig. 2. Distribution of scores (responses per 25 trials) for injected planarians

Fig. 2 presents the number of responses made by each worm in the test series. The mean for planarians injected with RNA from group CC was 8·2, that for the 'PC-injected' group was 1·8, and that for the NC-injected group was 1·6. By a Mann–Whitney U test[7], the group injected with RNA from classically conditioned worms was superior in performance to either of the other injected groups (P < 0·001). As Fig. 2 shows, the ranges of the two control groups, on one hand, and of the experimental group, on the other, did not overlap.

Several conclusions can be drawn from this experiment: First, classical conditioning in planarians is a distinct and valid phenomenon, and pseudo-conditioning effects, at least under the present conditions, are negligible. This finding corroborates that of Baxter and Kimmel[8], but in the present experiment the differences between groups were much more pronounced. Secondly, injection of the RNA preparation from group CC was sufficient to induce responsiveness to the light which could not be accounted for on the basis of nutrition, handling, or stimulation received by the donor group. Rather, the critical feature for both the conditioning effect and the RNA transfer effect was apparently the contiguity between the stimuli (light and shock). Since such contiguity is generally considered to be the defining characteristic of 'true' conditioning, the present data would seem to suggest that a specific learned response was transferred by way of the injection of the RNA preparation. This conclusion is strengthened by the fact that no shock was used during testing, and thus no new learning could have occurred.

We have conducted two supplementary experiments designed to ascertain whether RNA was in fact the effective transfer agent: (1) RNA extracted from CC and PC groups by the phenol method as previously described was further purified by 'Sephadex' chromatography (G-50 coarse beaded form; 1 mM magnesium acetate 5 mM *tris*-acetate, *p*H 7·2) and methylated albumin chromatography (step-by-step elution with 2·0 M sodium chloride, 0·05 sodium phosphate, *p*H 6·7). A significant difference in the levels of response was found between the two groups injected with these purified preparations (mean of experimentals, 7·8; mean of controls, 2·9; P < 0·001). (2) RNA from CC animals was degraded by treatment with 0·3 N sodium hydroxide (16 h at 37° C). Recipients (group 2) of this degraded RNA performed at about the same level as did recipients (group 3) of RNA from PC animals, and both were significantly lower in performance than recipients (group 1) of RNA from CC animals (mean of group 1, 8·4; mean of group 2, 4·7; mean of group 3, 5·7; group 1 versus group 3: $P < 0{\cdot}01$; group 1 versus group 2: $P < 0{\cdot}001$).

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II. The Influence of Prior Extinction on the Ribonucleic Acid Transfer Effect

A NUMBER of experiments, including the preceding article, appear to implicate RNA in memory functions. The most prevalent approaches attempt either to correlate RNA changes with a learning experience[1], or to transfer behavioural tendencies from one organism to another by injection of RNA[2]. These experiments and the relevant theory generally concern the acquisition (learning) of new behaviour patterns. Any adequate biochemical concepts of memory must also, however, account for the diminution or loss of learned behaviours which typically occurs in experimental extinction. Proponents of the RNA hypothesis might adopt either of two positions on this point: (*A*) experimental extinction acts to modify (either directly or indirectly) the RNA which was (hypothetically) modified during acquisition; or (*B*) experimental extinction results in behavioural decrements by some mechanism which does not involve changes in RNA. The latter position implies that any modifications in RNA produced during acquisition persist during and after extinction.

The present experiment attempts to assess these two hypotheses. As in the investigation described in the preceding article, one group of planarians was classically conditioned and a second group was pseudo-conditioned. A third group was classically conditioned and then experimentally 'extinguished' by repeatedly presenting the conditioned stimulus (CS) alone. RNA extracted from each of these three groups was injected into naïve planarians. Hypothesis *A* would predict that the group receiving RNA from 'extinguished' worms should be comparable in performance to the group receiving RNA from pseudo-conditioned worms; hypothesis *B* would predict that the group receiving RNA from 'extinguished' worms should be comparable with the group receiving RNA from classically conditioned worms.

Initially, 400 worms received classical conditioning trials with paired light and shock, and 200 worms received pseudo-conditioning trials with unpaired light and shock. The procedures followed those described in the preceding article: fifty exposures to light alone were followed by 250 training trials in sets of 25 trials. Four animals randomly selected from each group were observed individually during the last 25 trials: those from the CC group scored 11, 13, 13, 13; those from the PC group scored 0, 1, 3, 4.

The classically conditioned group was then divided into two equal groups. One of these groups (CCE) was given 200 presentations of light alone at 20-sec intervals, and a second session of 50 light-alone trials after a 1-h break. The other conditioned group (CC) and the pseudo-conditioned group (PC) were equated to group CCE in terms of handling and apparatus exposure—that is, these groups were placed in the apparatus but were not given presentations of light. Four animals selected at random from each of the three groups were then tested for 25 trials: CC animals scored 10, 11, 13, 13; PC animals scored 1, 2, 5, 5; and CCE animals scored 1, 2, 2, 5.

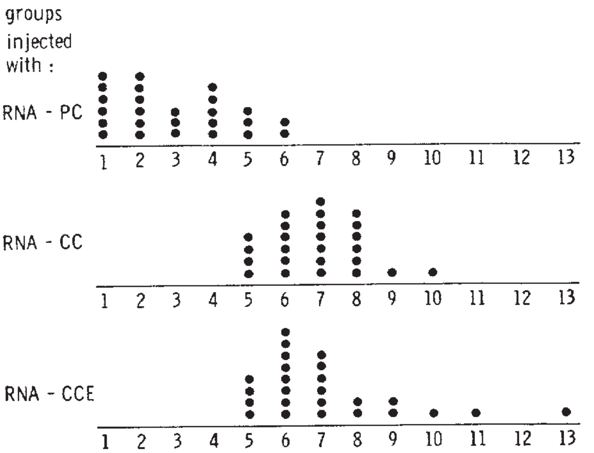

Fig. 1. Distribution of scores (responses per 25 trials) for injected planarians

RNA was then extracted (in blind fashion) from groups CC, PC and CCE by the phenol method as previously described and was further purified by 'Sephadex' chromatography (*G*-50 coarse beaded form; 1 mM magnesium acetate, 5 mM *tris*-acetate, *p*H 7·2). The RNA extracted from each of the three groups was injected into 25 naïve planarians which had previously been given 100 presentations of light alone. Each animal received 25 μl. of the RNA preparation. Injections were performed in blind fashion. Twenty-four hours later, each injected animal was given 25 presentations of light alone and was scored for number of responses (contractions). This testing was 'blind', and animals from the three groups were taken in a randomly interspersed order.

Fig. 1 presents the number of responses made by each worm on the test series. The mean for planarians injected with RNA from group CC was 6·9, that for worms injected with RNA from group CCE was 7·2, and that for worms injected with RNA from group PC was 3·0. The medians were, respectively, 7, 7, and 3. By a Mann-Whitney *U* test[3], the group injected with RNA from PC worms differed markedly from each of the other two injected groups ($P < 0{\cdot}001$). The latter two groups clearly did not differ.

Thus, the effects on naïve recipients of RNA from extinguished planarians were indistinguishable from the effects produced by RNA from conditioned planarians. One possible interpretation of these data is that spontaneous recovery might account for the level of response of the group injected with RNA from group CCE. Some subsidiary data force us to reject this possibility: five worms from group CCE were put aside following conditioning and subsequent extinction and were then given 25 test trials at the time that testing of the injected animals was conducted. The scores obtained were: 0, 1, 1, 1, 2. Clearly there was no spontaneous recovery of the conditioned response. In addition to substantiating our earlier findings, these results, then, suggest that experimental 'extinction', while abolishing the conditioned response in planarians, did not affect the RNA of the conditioned animals.

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